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Identification of Novel Dendritic Cell Populations in Normal Mouse Retina

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Abstract

Purpose—Whether tissue resident or infiltrating antigen-presenting cells (APCs) are involved in modulating immune responses in the retina and initiating inflammation is controversial. In this histologic study, the authors examine the retinas of mice strains with different susceptibility to experimental autoimmune uveoretinitis (EAU) for tissue resident APC.

Methods—Retinal wholemounts from normal and inflamed eyes of B10R III, C57BL/6, BALB/c, and ABH Biozii mice were immunostained for APC markers (33D1, CD11c, CD11b, major histocompatibility complex [MHC] class II, F4/80, CD80, CD86, CD205, mPDCA, B220, and GR1) and analyzed by confocal fluorescence microscopy using emission fingerprinting and three-dimensional reconstruction techniques. Hematoxylin and eosin-stained histologic sections were used to evaluate EAU disease scores and to assess outer blood retina barrier (retinal pigment epithelium [RPE]) structures.

Results—A population of 33D1⁺ cells was identified exclusively in the peripheral margins and juxtapapillary areas of the retina in normal, nonimmunized C57BL/6 adult mice. These cells were also MHC class II^{high}, and their location corresponded to sites of earliest inflammation in EAU. Numbers in the papillary area were very low (less than 10), but this region marked the predominant anatomic site for initiation of inflammation in this moderately susceptible strain. The distribution and phenotype of these cells within the retinas differed between mouse strains exhibiting different disease susceptibility. In EAU-resistant BALB/c mice, many more 33D1⁺ dendritic cells were present in the normal retina but were MHC class II^{low/-}. Conversely, no 33D1⁺ or MHC class II⁺ dendriform cells could be found in the normal retinas of highly EAU-susceptible B10.RIII mice.

Conclusions—A novel population of 33D1⁺ DCs was identified in normal mouse retina. The function of these cells remains to be defined, but increased numbers correlate positively with structural abnormalities in the RPE and increased resistance of the strain to EAU.

Tissue-resident dendritic cells (DCs) are essential to maintain immunologic homeostasis in most tissues.¹ Other regulatory mechanisms are thought to be invoked within the brain parenchyma and retina because typical DCs have not previously been identified in these tissues,² and, though lacking defined lymphatics, injected antigen can be traced to the cervical lymph node from both brain and retina.^{3,4} In the eye, afferent drainage from the

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anterior and posterior chambers is mainly to the blood of the aqueous venous plexus through the trabecular meshwork,⁵ leading to an altered or abnormal immune response to intraocular antigens.⁶ A minor drainage pathway has also been described through the ciliary body into the suprachoroidal space and then to the uveovortex or uveoscleral drainage vessels,⁷ and this pathway may allow soluble antigens from vitreous or aqueous to enter regional lymph nodes.^{3,8} Cellular traffic from the eye and brain are also not well defined. In the brain, DCs are thought to migrate to lymph nodes from the cerebrospinal fluid compartment rather than from the brain parenchyma,⁴ but, in the eye, clear evidence that iris and ciliary body resident macrophages and DCs are able to migrate to draining lymph nodes is lacking.^{3,9} The presence of the blood-retinal barrier (BRB) and a predominantly immunosuppressive intraocular microenvironment are also thought to contribute to the suppression of local immune responses to retinal antigens,¹⁰⁻¹² yet retinal inflammation is not uncommon.

How autoimmune retinal inflammation is initiated is not understood. Autoreactive T cells must be reactivated to induce disease, and the question is whether this takes place in the draining lymph nodes, the retina, or both.^{8,9} T-cell transfer studies indicate that only activated T cells can cross the BRB.^{13,14} Retinal antigen-specific T cells activated in vitro require restimulation in vivo, and it is assumed that they must encounter cognate antigen on antigen-presenting cells (APCs) within the retina to initiate retinal inflammation. Antigen passively accessing draining lymph nodes through the uveoscleral route might be expected to induce tolerance. Although DCs have been detected in normal uvea^{15,16} and the inflamed retina,¹⁷ to date there has been no definitive description of typical APCs in normal, quiescent retina.^{15,18} Over the years, several groups have attempted to identify potential retinal APCs. MHC class II⁺ microglia have been observed in postmortem human eyes¹⁹ and in some rodent studies, but constitutive expression is controversial.^{18,20} Nonhematopoietic cells have been discounted,^{10,21,22} and retinal astroglia (Müller) cells are also thought not to be involved.²³ Myeloid-derived CD45⁺ microglia are thought to be suppressive, and perivascular macrophages have poor antigen-presenting capability and are not absolutely essential for disease induction,^{18,20,24} so the identity and origin of any eliciting APCs remain obscure.

Improved retinal wholemount preparation techniques, availability of fluorescence-labeled monoclonal antibodies, and advanced confocal microscopes provide new opportunities for the detection of rare cells within complex deep tissue. In this study, we revisit the hypothesis that, as in other tissues, the retina contains populations of DC that regulate immune responses and maintain immune homeostasis.

Materials and Methods

Animals and EAU Induction

C57BL/6, B10.RIII, and BALB/c mice were supplied by the Medical Research Facility Department at the School of Medical Sciences, Aberdeen University. Adult mice weighed between 20 and 25 g. Fixed globes from ABH Biozzi mice were obtained from David Baker, University College London. The animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and under the regulations of the UK Animal License Act 1986. EAU was induced in C57BL/6 mice through subcutaneous injection of 500 μ g human interphotoreceptor retinoid-binding protein (IRBP) peptide 1 to 20 (GP₁HLFQPSLVLDMAKVLLD; Sigma-Genosys, Cambridge, UK) emulsified 1:1 with CFA (CFA, H37Ra; Difco Laboratories, Detroit, MI).²⁵ Mice were administered an additional intraperitoneal injection of 100 μ L (1.5 μ g) *Bordetella pertussis* toxin.²⁵ All animals used for EAU induction were 8 to 12 weeks old and weighed approximately 20 g. Control mice were immunized with irrelevant antigen (ovalbumin [OVA]; Sigma) plus adjuvant as negative controls. Pathologic features of EAU in C57BL/6

mice have been described elsewhere.²⁶ In this chronic model of EAU, retinal inflammation starts from day 14 postimmunization (pi), mild to moderate disease occurs around day 21 pi, and more severe inflammation develops by day 28 pi. BALB/c mice infected through the cornea with herpes simplex virus type (HSV)-1 develop inflammatory chorioretinitis²⁷ and in some experiments were used to compare autoimmune and nonautoimmune retinal inflammation.

Pathology of Normal and Inflamed Retina

Normal ($n = 6$) mice and day 16 to 28 pi ($n = 116$) C57BL/6 mice were killed, and their eyes were fixed in 4% neutral-buffered formalin and embedded in paraffin wax. The eyes were oriented in the blocks so that the sections passed longitudinally through the anterior chamber and posterior pole close to the optic nerve. At least three sections were cut from each eye, and the sections were stained using standard hematoxylin and eosin (H&E) methods and were scored for inflammation. The location of infiltrating inflammatory cells and the degree of inflammation (grades 1-5) were evaluated by at least two independent observers using a detailed histologic scoring system.²⁸ Anatomic locations were classified as juxtapapillary, including connective tissue of optic disk, inner limiting membrane, and border tissue of Elschnig; peripheral, defined as the retina from posterior pole to anterior margin; or pars plana, defined as the area from the anterior margin of the sensory retina, including collecting venules of the retina and ora serrata at the junction of the choroid and the ciliary body. Inflammation of these anatomic regions was scored as papillitis (optic nerve area), central retinitis (all retina excluding a 2 mm marginal rim), or peripheral marginal retinitis (restricted to a 1.0-mm rim of retina and ora serrata and encroaching the ciliary body).

Ablation and Reconstitution of Bone Marrow-Derived Retinal Cells

C57BL/6 mice 8- to 12-weeks old ($n = 6$) were irradiated with 8 Gy gamma ray. Ten million bone marrow cells from homozygous C57BL/6 mice expressing enhanced GFP (EGFP) under the control of a chicken b-actin promoter and CMV enhancer (obtained from Masaru Okabe, Osaka University, Osaka, Japan) were transplanted through the tail vein into irradiated mice. Mice were killed 6 and 12 weeks after reconstitution, and retinal wholemounts were prepared for confocal microscopy.

Immunofluorescence Confocal Microscopy of Retinal Wholemounts

Mice were killed by CO₂ inhalation, and the eyes were enucleated and fixed with 2% (wt/vol) paraformaldehyde in phosphate-buffered saline (Agar Scientific Ltd., Cambridge, UK). Retinas were dissected as previously described.^{29,30} In some experiments, retinas were cultured from 4 to 16 hours with 1 μ g/mL lipopolysaccharide (LPS; Sigma) or 1 μ g/mL LPS plus 100 ng/mL TNF- α (BD Biosciences, Oxford, UK) before fixation and staining. After washing, retinal tissues were blocked and permeabilized with 5% (wt/vol) bovine serum albumin (BSA) with 0.3% (vol/vol) triton in PBS at room temperature for 1 to 2 hours. Samples were then incubated overnight in the dark at 4°C with different combinations of anti-mouse antibodies. Antibodies used were fluorescein isothiocyanate (FITC)-conjugated anti-CD11c (HL3), FITC-conjugated anti-CD11b (M1/70), allophycocyanin (APC)-conjugated anti-CD8 α (53-6.7), APC-conjugated anti-GR-1 (Ly-6G; RB6-8C5), APC-conjugated anti-B220 (RA3-6B2), purified rat anti-DC marker (33D1, followed by FITC-conjugated anti-rat IgG), R-phycoerythrin (R-PE)-conjugated anti-I-A/I-E (M5/114), FITC-conjugated anti-CD80 (16-10A1), APC-conjugated anti-CD86 (GL-1; BD Biosciences); R-PE-conjugated anti-F4/80 (A3-1), and FITC-conjugated anti-CD205 (NLDC-145; all from Serotec, Oxford, UK); FITC-conjugated anti-mPDCA-1 (JF05-1C2; Miltenyi Biotec Ltd., Surrey, UK); and R-PE-conjugated anti-MD-1 (MD-113; Abcam Ltd., Cambridge Science Park, UK). After thorough washing, retinal tissues were flat mounted with vitreous side up (Vectashield; Vector Laboratory Ltd., Peterborough, UK) on clean glass slides. Retinal flat

mounts were examined under a confocal scanning laser microscope (LSM 510 META; Carl Zeiss, Göttingen, Germany) configured to eliminate autofluorescence and spectral overlap allowing precise discrimination between the fluorochromes imaged. Z-stacks of confocal images of retinal wholemounts were reconstructed and analyzed (Image Pro Plus; Media Cybernetics, MD). Cryosections of spleen and normal eye were used as positive control tissue and the showed expected distribution of staining by F4/80 and 33D1 antibodies.

Evaluation of Leukocyte Adhesion and Infiltration

To evaluate leukocyte adhesion and infiltration during the development of EAU, splenocytes from IRBP peptide-immunized mice were labeled in vitro with 10 $\mu\text{g/mL}$ calcein-acetoxymethyl ester (C-AM; Molecular Probes, Leiden, The Netherlands), at 37°C for 30 minutes. After extensive washing these cells were adoptively transferred (1×10^7 cells in 100 μL PBS) through the tail vein into syngeneic anesthetized mice. 30 Normal and immunized mice with early-stage EAU (day 16 pi; $n = 3$) were used. After 16 hours mice were injected with 2% Evans blue to label endothelium and to indicate BRB leakage, and retinal wholemounts were prepared and evaluated as described.

Results

Site of Early Retinal Inflammation

We first focused on the anatomic location of retinal inflammation in C57BL/6 mice with EAU at various times from disease onset through early to moderate disease using standard histologic sections. One hundred sixteen day 16 to approximately day 28 pi C57BL/6 mice (232 eyes) were examined. Among these, 146 (63%) eyes had retinal inflammation, and, of these, 34 (23.29%) had the optic disk structure in the field of view. When sections with and without optic disk were studied separately, a high incidence of inflammation in the peripheral margin retina (marginal retinitis) (Figs. 1A, 1C) and optic disk (papillitis; Figs. 1B, 1D) was noted, particularly in eyes with early or mild inflammation (grades 0.5-1.5; Figs. 1C, 1D), as has been reported by others.^{17,31} As disease progressed (grades 1.5-2.5 and grades 2.5-4.0), inflammation spread to other parts of the retina (Figs. 1C, 1D).

The preferential site of retinal inflammation in the areas of the peripheral margin retina and the optic disk was further confirmed by our adoptive transfer study in which fluorescence-labeled splenocytes from normal mice were adoptively transferred (intravenously) into syngeneic mice 16 days pi in IRBP-immunized mice (EAU onset). Confocal microscopy of retinal flat mounts revealed a focus of transferred cells adhering to vessels in the juxtapapillary areas (Fig. 2A). Substantial numbers of leukocytes were also seen infiltrating the peripheral retinal margin, with some cells also forming inflammatory foci within the collecting venules (Fig. 2B). Retinal vasculitis, a major pathologic feature of EAU, developed later in the disease process (days 21-25; data not shown). No signs of inflammation (cell adhesion, infiltration, and Evans Blue leakage) were observed in control OVA-immunized mice (Fig. 2C).³⁰ These data suggest that the optic nerve and peripheral marginal retina represent points of susceptibility to the onset of inflammation in this model of EAU and that the retina is not uniformly at risk.

Specific Location of MHC Class II⁺ Cells in Juxtapapillary and Retinal Marginal Areas of Normal Retina

To determine why the peripheral margin retina and the optic disk were preferential sites for early retinal inflammation, we examined these areas in normal mouse retina for the presence of DCs or other cells that could promote inflammation. Staining of wholemount retinas of normal adult C57BL/6 mice (8-12 weeks old) revealed low numbers (8.86 ± 2.85) of dendriform MHC class II⁺ cells per retina in the juxtapapillary area (Fig. 3A) and $54.14 \pm$

12.43 cells per retina in the marginal retinal area (Fig. 3B). The presence of these cells was age dependent. MHC class II⁺ dendriform cells were not detected in mice younger than 2 weeks (Figs. 3C-F), and the number increased with age and reached a plateau between 4 and 8 weeks of age (Fig. 3H). The limited number and highly restricted distribution of these cells was notable, with only the occasional MHC class II dendriform cell observed with these clearly defined areas. Reconstitution of irradiated adult C57BL/6 mice with syngeneic EGFP-expressing bone marrow cells indicated that repopulation of these MHC class II dendriform cells in adult retina took up to 6-12 weeks, suggesting these cells were able to turn over within the retina at a slow rate (Fig. 3G).

Double staining of wholemount retinas with anti-mouse CD31 antibody showed that in the peripheral margin retina, dendriform MHC class II⁺ cells were mainly located in the inner retinal vascular layer or slightly deeper (Figs. 4A, 4B). In the retina, CD31 is predominantly expressed by venules, and Fig. 4A shows that some MHC class II⁺ displayed perivascular arrangement around the retinal venules (Figs. 4A, 4B) but not around the arterioles (Fig. 4C).³⁰ These cells were distinct from the more numerous MHC class II⁺ cells of different sizes and shapes in the ciliary processes connected to the retinal margin (Figs. 3B, 4A). In the juxtapapillary area, MHC class II⁺ cells could be observed in close apposition to the optic nerve, with some processes wrapping around the optic disk and others spreading into the surrounding neural retina (Figs. 3A, 4C, 4D). Transverse views of the reconstructed Z-stack images revealed that these MHC class II⁺ cell processes penetrated the whole thickness of the retina surrounding the optic nerve (Fig. 4D).

Retinal MHC Class II⁺ Cells Are 33D1⁺ Dendritic Cells

Microglia can also express MHC class II antigens³²; hence, we performed double staining with a panel of myeloid- and APC-specific antibodies to phenotype the dendriform MHC class II⁺ cells. They were found to be weakly positive for CD11b (Fig. 5A) but negative for F4/80, CD11c, CD8 α , CD205, B220, GR1, mPDCA-1, CD86, CD80, and MD-1. In contrast, all MHC class II⁺ cells were strongly positive for the mouse DC-specific marker 33D1^{33,34} (Fig. 5B). Ex vivo stimulation of retinal tissue with 1 μ g/mL lipopolysaccharide or LPS plus 100 ng/mL TNF- α for 4 to 16 hours showed no enhancement of any of the cell surface markers tested (data not shown). Dual staining of mouse spleen sections showed that 33D1 was not expressed on F4/80 macrophages (Fig. 5C).

We next examined the distribution of MHC class II⁺ cells in early EAU. Their numbers increased as disease progressed. In day 18 pi C57BL/6 mice, significantly more MHC class II⁺ cells were observed in the juxtapapillary (86 ± 9.92 vs. 40 ± 9.88 ; $n = 6$; $P < 0.01$) and peripheral retinal areas (249.3 ± 21.32 vs. 162.0 ± 24.43 ; $n = 6$; $P < 0.05$) than at 16 days pi. Many of the cells showed perivascular contact (Figs. 5D, 5E), and it was significant that perivascular dendriform MHC class II⁺ cells were observed only around venules, not arterioles (Fig. 5E). This finding correlates with our previous observations that BRB breakdown and retinal infiltration are largely confined to venules even in advanced EAU in mice^{30,35} and with the observations of McMenamin et al.³⁶ in rat EAU in which high endothelial-like changes to the ultrastructure of the vessels occur. In retinas with more severe disease, massive numbers of MHC class II⁺ dendriform cells were detected throughout the inflamed retina (Fig. 5F). These MHC class II⁺ dendriform cells were also positive for the 33D1 dendritic cell marker (Fig. 5F) and weakly positive for CD11c (data not shown). CD3 T cells were observed clustering with these MHC class II and 33D1 double-positive DCs (Fig. 5D). No vascular endothelial cells were positive for the MHC class II molecule (Fig. 5H). No change was observed in the number or the phenotype of these retinal MHC class II⁺33D1⁺ cells in OVA-immunized mice compared with nonimmunized mice (data not shown).

Retinal 33D1⁺ Dendritic Cells in Other Mouse Strains

Having identified retinal 33D1⁺MHC class II⁺ dendritic cells in C57BL/6 mice, we next examined retinal dendritic cells in other strains of mice with different susceptibilities to EAU induction. Surprisingly, in the highly EAU-susceptible B10R III mice, no 33D1 or MHC class II-expressing cell was observed in any area of normal retina (Fig. 6A), whereas abundant 33D1⁺ cells (502.1 ± 79.39 per retina; $n = 6$) were detected in the normal retina of EAU-resistant BALB/c mice with no specific distribution. However, most of these 33D1⁺ cells were MHC class II⁻ (Fig. 6B). Only a few cells expressed MHC class II, but this was at a very low level. The presence of these 33D1⁺ cells in BALB/c mice was not an artifact of the albino phenotype because 33D1⁺ cells could not be found in the retinas of EAU-susceptible ABH Biozzi mice, though dendriform 33D1⁺ MHC class II⁺ cells were present in the ciliary body (Fig. 6C).

Although no 33D1⁺MHC class II⁺ dendritic cells were observed in normal retinas of B10R III mice, a large number of MHC class II and 33D1 double-positive cells were detected in the inflamed retina (Fig. 6D) of B10.RIII mice (induced by immunization with IRBP peptide 161-18030). Given that BALB/c mice are resistant to EAU, we examined wholemount retinas from BALB/c mice with ocular herpes simplex infections. The retinas were inflamed and hemorrhagic, and inflammatory infiltrates of 33D1⁺ cells were present throughout the retina, notably in the optic disk area, but no increase in MHC class II expression was observed (Fig. 6E). In contrast to 33D1⁺ cells in EAU, the cells were not dendriform but were rounded or elongated, suggesting a more motile phenotype (Fig. 6E).

BRB in Murine Juxtapapillary and Pars Plana Region of Marginal Retina

To understand the specific location of the 33D1⁺MHC class II⁺ cells in normal C57BL/6 mice, we revisited the retinal anatomy, in particular the BRB barrier. The RPE and Bruch membrane form the outermost layer of the BRB barrier, and its margins represent points at which the barrier may be vulnerable. The sensory retinal margin is directly connected to the epithelial cells of the ciliary body at the pars plana and in the juxtapapillary area, and the RPE cell layer terminates at the optic nerve sheath and segregates the neuroretina from the underlying choroidal tissue (Fig. 7A). In some sections of C57BL/6 mouse retina, we observed that the RPE cell layer did not contact the edge of the optic nerve sheath, leaving the neuroretinal tissue directly in contact with the underlying choroid (Fig. 7B). Therefore, we hypothesized that the lack of physical barriers among ciliary epithelial cells at the marginal retina and between the neuroretina and the choroid in the juxtapapillary area may contribute to the presence of 33D1⁺ dendriform cells in these areas. We compared the incidence of RPE discontinuity at the optic nerve sheath in C57BL/6 mice with the incidence in BALB/c mice and B10.RIII mice. The results supported this hypothesis because the RPE barrier was largely intact in the highly susceptible B10.RIII strain, which has few or no 33D1⁺ retinal cells, whereas in the highly resistant BALB/c strain, which has many 33D1⁺ retinal cells, a high percentage (more than 75%) of eyes showed major structural abnormalities in the RPE (Figs. 7C-E). In the optic disk area of BALB/c mice, neural retinas were in direct contact with optic nerve (Fig. 7C). In the peripheral retina, RPE morphology was abnormal in some areas, with the normally plump cells becoming highly attenuated (Fig. 7D). RPE morphology in BALB/c mice was not simply an artifact of nonpigmented RPE because the RPE of EAU-susceptible Biozzi ABH mice³⁷ is structurally comparable with that in pigmented strains, and only 40% of retinas showed morphologic evidence of structural abnormalities in the RPE barrier. Figure 7E shows that the incidence of RPE barrier anatomic abnormality correlates negatively with EAU susceptibility but that it may correlate positively with the presence of 33D1⁺ cells in the retina.

Discussion

In this study, we have identified a small population of phenotypically distinct DCs in the mouse retina that may be analogous to recently described bone marrow-derived APCs in the brain.³⁸ To date these cells have remained unobserved through conventional microscopy, possibly because of their relatively low numbers (50-70 per retina in C57BL/6 mice), their variable phenotype, and their highly dendriform morphology. Given the relative abundance of DCs in the iris and the ciliary body, their variable presence in the retina of normal mice strains is notable.¹⁶ The putative retinal APCs in C57BL/6 and BALB/c mice were identified as DCs by their distinctive morphology, together with high expression of 33D1 antigen. The nature and function of the 33D1 antigen is unclear, but it specifically reacts only with a subset of mouse DCs.^{33,34,39,40} The origin of these cells in normal retina is unknown, but, because of their anatomic location, it is possible that they migrated to the retina from the choroid, the ciliary body, and the meninges. This is supported by the positive correlation between DC number and RPE structural abnormality found in this study. It is also possible that the lack of DCs in the retinas of normal B10 RIII mice results from a lower ability of the cells to migrate through the RPE or from the ciliary body or the peripapillary meninges in this strain, possibly as a result of variations in chemokine expression or other mediators of chemotaxis. Structural abnormalities in the BRB at the optic nerve are well recognized and have been demonstrated using fluid tracers at the RPE⁴¹ and the microvasculature levels.⁴² In a recent report,⁴³ autoantigens, including the retinal antigens S-antigen and IRBP and the CNS autoantigens MOG (myelin oligodendrocyte glycoprotein) and PLP, were found to be chemoattractants for immature DCs expressing CXCR5 or CXCR3, providing a mechanism for active recruitment of these cells, particularly in retinas in which the RPE appears attenuated or discontinuous. The phenotype of these DC differs from that of resident choroidal DCs reported by McMemamin,¹⁶ but this could reflect an adaptation to the immunosuppressive retinal environment. Another possibility is that they are differentiated from retinal microglia or perivascular macrophages.⁴⁴ However, CD11b⁺/MHC class II^{low/-} microglia could be clearly distinguished from 33D1⁺CD11b^{low/-} MHC class II⁺ dendriform cells (Fig. 5A). Factors secreted in the choroidal tissue and ciliary body reaching the neuroretina through structural abnormalities in the BRB at the optic nerve⁴¹ or from the highly vascularized ciliary body may control differentiation into MHC class II⁺ 33D1⁺ APCs. Why these dendriform perivascular cells are present only in association with retinal venules and not in arterioles cannot be explained by either of these hypotheses but may be consistent with the fact that the postcapillary venule is the site of initial breakdown of vascular integrity and of the angiogenic response after capillary occlusion.

The function of mouse retinal DCs observed in this study remains elusive and may be determined by the integrity of the BRB and the response of the individual to local immunosuppressive and microenvironmental factors, as determined by genetic polymorphisms. It is also possibly linked to the regulation of APC gene expression as a result of different levels of immunosuppressive mediators within the ocular microenvironment of different mouse strains. To examine this possibility, laser microdissection techniques that will enable us to examine these cells at the molecular level directly *ex vivo* are now planned. The anatomic location of these cells in C57BL/6 mice coincides with the sites of earliest inflammation; significantly, the perivascular distribution is restricted to venules, the preferential site for leukocyte extravasation in EAU.^{17,30,45} However, because they do not express costimulatory molecules even after LPS and LPS + TNF- α treatment, functionally these APC will be restricted to reactivation of antigen-primed T cells, at least within the retinal microenvironment. This prediction is in accord with Gregerson's observations that CD45⁺ retinal microglia and perivascular cells had limited ability to reactivate T cells.^{18,24} Activated T cells of any specificity are able to enter the

normal retina.^{13,14,46} It has been suggested that retinal autoimmunity in humans may occur as a result of pathogenprimed T cells cross-reacting with retinal antigens through a process of molecular mimicry.^{47,48} It should be noted that MHC class II⁺ dendriform cells have also been observed in the normal postmortem wholemount human retina.⁴⁹ Cells were frequently associated with blood vessels and were most abundant at the peripheral margins of the retina. Determining the role of this new population of retinal DCs will be important for understanding the triggers for human posterior uveoretinitis.

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Figure 1. Histopathologic features of retinal inflammation in experimental autoimmune uveitis. Photoreceptor rod outer segments and inner and outer nuclear layers are largely unaffected in early inflammation. **(A)** Inflammation at anterior margin of retina, showing vasculitis around collecting tube venule, vitreous infiltration, and infiltration of choroid and ciliary body. Original magnification, $\times 125$. **(B)** Inflammation in optic disk, showing moderate leukocyte infiltration in the optic disk and vitreous, with some involvement of adjacent retina. Original magnification, $\times 62.5$. **(C-D)** Correlation between grade of disease and anatomic location of inflammation. **(C)** Retinal inflammation with optic disk in the section. **(D)** Retinal inflammation without optic disk in the section. Sections were scored by two observers, and similar results were obtained. Data presented as percentage of inflamed eyes within each grade spectrum showing the specific inflammatory pattern from one scorer. Ch, choroid; CB, ciliary body; CT, collecting tube; PP, pars plana; JP, juxtapapillary region of retina; ON, optic nerve; OD, optic disk; R, retina; RPE, retinal pigment epithelium.

Figure 2.

Leukocyte adhesion and infiltration in retinal wholemounts. Splenocytes from syngeneic mice were in vitro labeled with C-AM and adoptively transferred into day 16 pi EAU-immunized mice (**A-B**) or OVA-immunized mice (**C**). Sixteen hours later, mice were injected with 50 μ L Evans Blue, and retinal wholemounts were prepared for confocal microscopy. Early leukocyte adhesion and infiltration were observed in the juxtapapillary area (**A**, *arrowhead*) and the collecting tube of the peripheral retina (**B**, *open arrow*) in day 16 pi EAU mice. No evidence of inflammation was observed in control OVA-immunized mice (**C**). Bar, 100 μ m.

Figure 3.

MHC class II⁺ cells in the juxtapapillary and peripheral retina of normal nonimmunized mice. Wholemount retinas from different ages of mice were stained for MHC class II and observed by confocal microscopy. (A, C, E) Juxtapapillary area. (B, D, F) Peripheral retina at margin between retina (R) and ciliary body (CB). Populations of highly dendriform MHC class II⁺ cells were observed in an 8-week-old mouse retina (A, B). These cells were only observed around the optic disk (A) and at the junction of the neural retina and ciliary body (B). No MHC class II⁺ cells could be detected in the retina of a 1-week-old mouse in either location (C, D). Some MHC class II⁺ cells could be detected in the retina from 2 weeks of age (E, F), again located specifically in the juxtapapillary and marginal retina. Scale bar, 50 μm . (G) Retina from an adult mouse 12 weeks pi and reconstitution with GRP⁺ bone marrow cells shows two GFP MHC class II double-positive cells in the retinal margin area.

(H) MHC class II⁺ cells in the juxtapapillary and peripheral retina increases significantly with age. Data are expressed as mean $\pm$ SD. $n = 6$ in 1-week, 2-week, and 4-week groups; $n = 20$ in 8-week group. R, retina; CB, ciliary body; OD, optic disk.

Figure 4.

Precise anatomic location of MHC class II⁺ cells in normal retina. Wholemound retinas ($n = 6$) were double stained with anti-mouse CD31 (*green*) and MHC class II⁺ (*red*) and were analyzed by confocal microscopy. Venules were identified by their anatomic location within the retinal vascular tree, comparative vessel diameter, and predominant expression of CD31. Z-stacks (65-100 μm) of the whole thickness of the retina were taken and reconstructed to the front view (**A**, **C**) or side view (**B**, **D**) for image analysis. (**A-B**) MHC class II⁺ cells were observed in the layer between inner and outer retinal vasculatures and surrounding the collecting tube in the peripheral retina. (**C-D**) MHC class II⁺ cells were observed surrounding the optic nerve and extending throughout the entire retinal thickness in the juxtapapillary area.

Figure 5.

Phenotype of MHC-class II⁺ cells. (A-B) Wholemount retinas ($n > 4$) from normal nonimmunized C57BL/6 mice were double stained with anti-mouse CD11b FITC (A, green) or mouse DC maker 33D1 FITC (B, green) and MHC class II PE (red). (C) Spleen sections from a normal mouse were stained for 33D1-FITC and F4/80 PE using a similar protocol for retinal wholemounts. (D-H) Wholemount retinas ($n > 6$) from day 16 to 28 pi EAU mice were double stained for CD31 FITC (D, E, H) or mouse DC marker 33D1 FITC (F) or CD3 FITC (G) and MHC class II PE and were analyzed by confocal microscopy. Increased numbers of MHC class II⁺ perivascular cells compared with normal controls were observed

in the venules of the peripheral margin (**D**) and the juxtapapillary area (**E**). (**F**) All MHC class II⁺ cells stained positively for mouse DC marker 33D1. (**G**) CD3 T cells were observed clustering with MHC class II⁺ cells (*open arrows*). (**F**) Vascular endothelial cells stained negatively for MHC class II molecules. a, arteriole; v, venule; CB, ciliary body.

Figure 6.

Mouse retinal DCs in other strains of mice. B10.RIII retina (**A**), BALB/c retina (**B**), and ABH Biozzi retina (**C**) were double stained for 33D1 (*green*) and MHC class II (*red*). Each panel shows single-channel collection and a merged image to show colocalization of signal. (**A**) No 33D1⁺ or MHC class II⁺ cells were observed in any B10.RIII retinas. (**B**) A large number of 33D1⁺ cells were detected in the retina of BALB/c, but they were MHC class II negative. (**C**) 33D1⁺ MHC II⁺ cells were observed in the ciliary body but not in the retina of an ABH Biozzi mouse. (**D**, **E**) Retinas from day 12 pi IRBP-immunized B10R III mice (**D**) or BALB/c mice with acute ocular herpes simplex infection were double stained for 33D1 FITC and MHC class II PE and observed by confocal microscopy. (**D**) All MHC class II⁺ cells stained positively for 33D1 in inflamed B10.RIII mouse retina. (**E**) Increased numbers of 33D1⁺ cells over control noninfected eyes (**B**) were observed and displayed altered

morphology compared with control and remained MHC class II negative. OD, optic disk. All images represent retinal wholemounts from 3 to 6 mice in each group.

Figure 7.

Outer blood retina barrier of normal retinal juxtapapillary areas. Eyes from normal nonimmunized mice were processed for hematoxylin and eosin (H-E) staining. (A-B) Eyes are from C57BL/6 mice and (C-D) from BALB/c mice. Juxtapapillary retinas with optic nerve are shown (A-C). Note (A) the RPE layer ends at the optic nerve sheet, whereas (B) there is a gap between the RPE and optic nerve sheet (*open arrow*). (C) Anatomic abnormality is more pronounced, showing large gaps in RPE (*arrow*) with extensive overlap between neural retina and optic nerve sheath. In the peripheral retina, RPE cells often appear highly attenuated or even absent, allowing close apposition between vascular choroid and neural retina (D, *arrow*). Histogram (E) showing the percentage of mouse eyes ($n > 40$; $n = 12$ for Biozzi mice) from each strain that show structural abnormality at the RPE/optic nerve

sheath. Ch, choroid; ON, optic nerve; RPE, retinal pigment epithelium. Original magnification, $\times 62.5$.